AN AMINO ACID SEQUENCE IN THE ACTIVE SITE OF LIPOAMIDE DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHILUS*

Leonard C. PACKMAN and Richard N. PERHAM
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

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1. Introduction

Lipoamide dehydrogenase (EC 1.6.4.3) is the flavoprotein component of the 2-oxoacid dehydrogenase multienzyme complexes in mammalian and bacterial systems. The enzyme is a dimer of seemingly identical polypeptide chains (M_r 54 000–56 000 according to the source) and there is evidence to suggest that the lipoamide dehydrogenases of the pyruvate and 2-oxoglutarate dehydrogenase complexes are identical [1–3].

Each polypeptide chain of the lipoamide dehydrogenases from *Escherichia coli* and pig heart contains a single disulphide bridge which undergoes alternate reduction and oxidation during the catalytic action of the enzymes. In both enzymes, the 2 cysteine residues forming the intrachain loop are separated by 4 amino acids and are found to inhabit highly homologous sequences [4,5]. A further homologous sequence has been reported for the redox-active disulphide bridge of glutathione reductase (EC 1.6.4.2) [6,7], a flavoprotein with a similar catalytic mechanism. However, no homology in disulphide bridge size or sequence is visible for thioredoxin reductase (EC 1.6.4.5) of *E. coli*, a third flavoprotein of this type [8,9]. One interpretation of this evidence would be that lipoamide dehydrogenase and glutathione reductase share a common ancestral gene [3], a view supported by further analysis of the amino acid sequences of pig heart lipoamide dehydrogenase and human erythrocyte glutathione reductase [10].

Here, we describe a simple technique for isolating the tryptic peptide derived from the redox-active disulphide bridge of lipoamide dehydrogenase from *Bacillus stearothermophilus* and show that its amino acid sequence is closely related to that from *E. coli* lipoamide dehydrogenase.

2. Materials and methods

Pyruvate dehydrogenase multienzyme complex was purified from *B. stearothermophilus* [11]. Amino acid analysis, thin-layer electrophoresis and chromatography on polygram SilG silica plates (Camlab), performic acid oxidation and dansyl-Edman sequencing were performed as in [12].

Polyacrylamide gel electrophoresis of proteins was performed in the presence of 0.1% sodium dodecylsulphate (SDS) [13] and in the absence of detergent (phosphate-buffered system of [14] omitting SDS).

3. Results

3.1. *Purification of lipoamide dehydrogenase*

Analysis of a sample of freshly prepared pyruvate dehydrogenase complex by SDS–polyacrylamide gel electrophoresis showed the presence of 4 polypeptide chains with app. M_r 42 000 (E1α), 36 000 (E1β), 57 000 (E2), 54 000 (E3) [11]. After several months storage of the complex at 4°C, proteolytic degradation of the E1α and E2 components was observed but the lipoamide dehydrogenase (E3) component remained intact and catalytically active. The latter component was purified as follows.

The enzyme complex was subjected to limited proteolysis with trypsin and fractionated by gel filtration on Sepharose CL-6B [15]. The lipoamide dehydrogenase-containing peak was further fractionated on hydroxylapatite [11] to yield a homogeneous preparation of lipoamide dehydrogenase as judged by polyacrylamide gel electrophoresis in the presence and absence of SDS.

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3.2. S-*Carboxymethylation of lipoamide dehydrogenase in the absence of reductant*

Lipoamide dehydrogenase (10 mg, 185 nmol) was equilibrated at 20°C under N_2 in 2 ml 0.2 M Tris–HCl (pH 8.5) containing 1 mM NAD^+ and 2 mM EDTA. Iodoacetic acid was added to 10 mM final conc. and the temperature was raised to 37°C. Crystals of Aristar guanidine hydrochloride were added to the mixture with stirring under N_2 until the solution was saturated. Incubation was continued in the dark for 4 h at 37°C and the modified enzyme was then dialysed exhaustively in the dark at 4°C against 50 mM ammonium bicarbonate. The carboxymethylated protein remained soluble during dialysis, retaining a large proportion of bound FAD. The protein was recovered by lyophilization, and resuspended by sonication in 1 ml 25 mM ammonium bicarbonate.

3.3. *Isolation of disulphide-bridge peptide*

The resuspended protein (0.85 ml, 10 mg/ml) was digested with 2% (w/w) TPCK-trypsin for 6 h at 37°C and then freeze-dried. The salt-free tryptic peptides were resuspended in 1 ml 8% (v/v) acetic acid, 2% (v/v) formic acid (pH 2 buffer). A small sample (10 μl) of the digest was subjected to diagonal electrophoresis at pH 6.5. Performic acid vapour (2 h, 23°C) was used between steps of electrophoresis to cleave any disulphide bridge present in the digest [12]. Staining the dried plate with ninhydrin–cadmium revealed a major ninhydrin-positive spot (peptide SPB-1) lying off the diagonal; after oxidation the electrophoretic mobility [16] of the peptide changed from +0.14 to −0.14 (rel. asp. −1.0).

The suspension of tryptic peptides in pH2 buffer contained a small insoluble fraction, which was removed by centrifugation. The clear supernatant was applied to an SP-Sephadex column (3.2 cm × 2 cm diam., 10 ml bed vol.) equilibrated with pH 2 buffer. The column was washed with 15 ml of the same buffer. FAD failed to bind to the column and emerged at the void volume. The column was developed with a series of 4 successive linear gradients (30 ml) at 0.5 ml/min flow rate. The gradients (I–IV) were generated by mixing 15 ml each of 2 buffers: (I) buffers (a,b); (II) buffers (b,c); (III) buffers (c,d); (IV) buffers (d,e); where (a) is pH 2 buffer, (b) is 5% (v/v) acetic acid 0.06 M pyridine (pH 3.5), (c) is 5% (v/v) acetic acid, 0.1 M pyridine (pH 3.65), (d) is 5% (v/v) acetic acid, 0.2 M pyridine (pH 4.10) and (e) is 0.25% (v/v) acetic acid, 0.62 M pyridine (pH 6.3). The column was finally washed with 30 ml 5% (v/v) triethylamine–acetic acid (pH 10.6).

A sample (50 μl) of each fraction (2 ml) was analysed by electrophoresis on silica plates at pH 6.5. A further 50 μl sample was similarly treated except that the applied peptide was oxidized with performic acid vapour before electrophoresis. The thiol-containing peptide, SPB-1, was detected by virtue of its altered mobility after oxidation. Fractions containing peptide SPB-1 (which had been eluted by gradient III) were pooled and freeze-dried. A tryptophan-specific stain [12] applied to the electrophoretogram of the unoxidized peptide suggested that peptide SPB-1 did not contain this amino acid.

The freeze-dried fraction was treated with performic acid [17], freeze-dried, and resuspended in 1 ml pH 2 buffer. Electrophoretic analysis confirmed complete oxidation of peptide SPB-1. The peptide fraction was rechromatographed on the SP-Sephadex column, equilibrated with pH 2 buffer, under conditions identical to those employed in the first separation. Oxidized peptide SPB-1 failed to bind to the column and emerged at the void volume whereas the remaining peptides chromatographed as before.

Table 1
Properties of peptide SPB-1

Amino acid	Mol/mol peptide	From sequence
Cya	1.7	2
Asx	2.0	2
Thr	<0.2	–
Ser	1.1	1
Glx	0.5[a]	–
Pro	1.2	1[b]
Gly	3.8	4
Val	2.0	2
Ile	0.9	1
Leu	1.7	2
Lys	0.9	1

Electrophoretic mobility at pH 6.5 [16]: +0.14 before oxidation, −0.14 after oxidation

Ninhydrin/cadmium stain: Yellow, slowly turning to orange

Sequence: $\underrightarrow{\text{Gly}}$–$\underrightarrow{\text{Asn}}$–$\underrightarrow{\text{Leu}}$–$\underrightarrow{\text{Gly}}$–$\underrightarrow{\text{Gly}}$–$\underrightarrow{\text{Val}}$–$\underrightarrow{\text{Cya}}$–$\underrightarrow{\text{Leu}}$–$\underrightarrow{\text{Asn}}$–$\underrightarrow{\text{Val}}$–$\underrightarrow{\text{Gly}}$–$\underrightarrow{\text{Cya}}$–$\underrightarrow{\text{Ile}}$–$\underrightarrow{\text{Pro}}$–$\underrightarrow{\text{Ser}}$–$\underrightarrow{\text{Lys}}$

[a] Glutamic acid appeared on the amino acid analyser chromatogram as an aberrantly shaped peak; Dns-Glu was not observed at any step of the sequence analysis

[b] Dns-Pro obtained in low yield: see text

($\underrightarrow{\quad}$) Residue established by dansyl-Edman degradation

3.4. *Amino acid sequence of peptide SPB-1*

Peptide SPB-1 (table 1) was isolated in 30–40% yield and sequenced by the dansyl-Edman method [18]. Dns-cysteic acid, although identified unambiguously, was obtained in poor yield. Proline (residue 14) was placed in the sequence by analysis; Dns-proline was detected only in trace amounts at this step of the degradation, probably because the hydrolysis time (16 h) of the dansylated peptide was too long to produce Dns-proline in high yield [19]. The electrophoretic mobility of the oxidized peptide suggested that both aspartic acid residues were present in the peptide as amides. The results are summarized in table 1.

4. Discussion

Peptide SPB-1 was isolated by making use of the change in net charge which could be introduced by oxidation of the intrachain disulphide bridge. Such a two-step chromatographic method provided a rapid and convenient means of isolating the desired peptide in high yield from limited amounts of material. Use of the diagonal electrophoresis technique would have achieved the same result but probably in much lower yield.

The sequence of peptide SPB-1 is similar to those of corresponding peptides from other enzymes (table 2). Differing only at the N-terminus and the third residue (deletion), the *B. stearothermophilus* sequence is perhaps most closely related to that of *E. coli* lipoamide dehydrogenase. In evolutionary terms, it is not surprising that 2 bacterial species of protein should appear more homologous than a bacterial and mammalian species. However, the pyruvate dehydrogenase complexes from *B. stearothermophilus* and mammalian sources, of which lipoamide dehydrogenase is a component, bear many common structural features (subunit M_r, symmetry, lipoic acid content) which are not shared by the *E. coli* complex [11,22,23]. These differences may have subtle underlying causes which will only become apparent when further analysis of primary structure has been done. The sequence homology found for the lipoamide dehydrogenases indicates that they belong to the growing list of proteins isolated from thermophiles that have structures closely related to those of their mesophilic counterparts (e.g., [24,25]).

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Table 2
Amino acid sequences around redox-active disulphide bridges

Lipoamide dehydrogenase[a]		
(a) Pig heart	T V C I E K B Z T L G G T C L N V G C I P S K	[5,20]
(b) *E. coli*	Y N T L G G V C L N V G C I P S K	[4]
(c) *B. stearothermophilus*	G N L G G V C L N V G C I P S K	
Glutathione reductase[a]		
(a) Human erythrocytes	A A V V E S H K L G G T C V N V G C V P K K	[6,21]
(b) Yeast	L G G T C V N V G C V P K	[7]
Thioredoxin reductase		
E. coli	A C A T C D G F	[9]

[a] Sequences of tryptic peptides

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